PCT/CH 20 04 / 000738

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Die beiliegenden Akten stimmen überein mit den ursprünglichen Unterlagen der auf den nächsten Seiten bezeichneten, beim unterzeichneten Amt als Anmeldeamt im Sinne von Art. 10 des Vertrages über die internationale Zusammenarbeit auf dem Gebiet des Patentwesens (PCT) eingegangenen Patentanmeldung.

Attestation

Les documents ci-joints sont conformes aux pièces originales relative à la demande de brevet spécifiée aux pages suivantes, déposées auprès de l'Office soussigné, en tant qu'Office récepteur au sens de l'article 10 du Traité de coopération en matière de brevets (PCT).

Confirmation

It is hereby confirmed that the attached documents are corresponding with the original pages of the international application, as identified on the following pages, filed under Article 10 of the Patent Cooperation Treaty (PCT) at the receiving office named below.

Bern, 22. November 2004

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Swiss Federal Intellectual Property Institute

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Administration des brevets
Patent Administration

Rolf Hofstetter

Anmeldeamtsexemplar

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

PCT/CH 03 / 00846
International Application No.

23. Dez. 2003 (23. 12. 03)
International Filing Date

RO / CH - Internationale Anmeldung PCT
Name of receiving Office and "PCT international Application"

Applicant's or agent's file reference
(if desired) (12 characters maximum) P142062 METB

Box No. I TITLE OF INVENTION
Method for isolating cells from mammary secretion

Box No. II APPLICANT ☐ This person is also inventor

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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This person is applicant for the purpose of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

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Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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State *(that is, country)* of nationality: Australia

State *(that is, country)* of residence: Australia

This person is applicant for the purpose of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is herby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

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☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Bestätigungskopie

PCT/CH 03 / 00846

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

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- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

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State *(that is, country)* of nationality: Australia

State *(that is, country)* of residence: Australia

This person is applicant for the purpose of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:

- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

State *(that is, country)* of nationality:

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This person is:

- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purpose of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:

- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office:

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Box No. V DESIGNATION OF STATES *Mark the applicable check-boxes below; at least one must be marked.*

The following designations are hereby made under Rule 4.9(a):

Regional Patent

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Check-boxes below reserved for designating States which have become party to the PCT after issuance of this sheet:

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Precautionary Designation Statement: In addition to the designation made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

PCT/CH 03 / 00846

Box No. VI PRIORITY CLAIM

The priority of the following earlier application(s) is hereby claimed:

Filing date of earlier application *(day/month/year)*	Number of earlier application	Where earlier application is:		
		National application: country	regional application:* regional Office	international application: receiving Office
item (1)				
item (2)				
item (3)				
item (4)				
item (5)				

☐ Further priority claims are indicated in the Supplemental Box.

The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of this international application is the receiving Office)* identified above as:

☐ all items ☐ item (1) ☐ item (2) ☐ item (3) ☐ item (4) ☐ item (5) ☐ Other, see Supplemental Box

** Where the earlier application is an ARIPO application, indicate at least one country party to the Paris Convention for the Protection of Industrial Property or one Member of the World Trade Organization for which that earlier application was filed (Rule 4.10(b)(ii)):* .....

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of the International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA / EPA

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/,month/year)* Number Country *(or regional Office)*

Box No. VIII DECLARATIONS

The following **declarations** are contained in Boxes Nos. VIII (i) to (v) *(mark the applicable check-boxes below and indicate in the right column the number of each type of declaration):*

		Number of Declarations
☐ Box No. VIII (i)	Declaration as to the identity of the inventor	:
☐ Box No. VIII (ii)	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	:
☐ Box No. VIII (iii)	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	:
☐ Box No. VIII (iv)	Declaration of inventorship (only for the purposes of the designation of the United States of America)	:
☐ Box No. VIII (v)	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	:

 PCT/CH 03 / 00846

Box No. IX CHECK LIST; LANGUAGE OF FILING

This international application **contains:**

(a) **in paper form,** the following number of sheets:

request (including declaration sheets):		5
description (excluding sequence listing part)	:	16
claims	:	5
abstract	:	1
drawings	:	8
Sub-total number of sheets	:	35
sequence listing	:	
tables related thereto	:	
(for both, actual number of sheets if filed in paper form, whether of not also filed in computer readable form; see (b) below)	:	
Total number of sheets	:	35

(b) ☐ **only in computer readable form** (Section 801(a)(i))
- (i) ☐ sequence listings
- (ii) ☐ tables related thereto

(c) ☐ **also in computer readable form** (Section 801(a)i(i))
- (i) ☐ sequence listings
- (ii) ☐ tables related thereto

Type and number of carriers (diskette, CD-ROM, CD-R or other) on which are contained the
- ☐ sequence listings:
- ☐ tables related thereto:

(additional copies to be indicated under items 9(ii) and/or 10(ii), in right column)

This international application is **accompanied by** the following item(s) *(march the applicable check-boxes below and indicate in right column the number of each item):*

		Number of items
1. ☒	fee calculation sheet :	1
2. ☐	original separate power of attorney :	
3. ☐	original general power of attorney :	
4. ☐	copy of general power of attorney; reference number, if any: ..... :	
5. ☐	statement explaining lack of signature :	
6. ☐	priority document(s) identified in Box No. VI as item(s): ..... :	
7. ☐	translation of international application into *(language):* ..... :	
8. ☐	separate indications concerning deposited microorganism or other biological material :	
9. ☐	sequence listing computer readable form *(indicate also type and number of carriers)*	
(i) ☐	copy submitted for the purposes of international search under Rule 13*ter* only (and not as part of the international application):	
(ii) ☐	*(only where check-box (b)(i) or (c)(i) is marked in left column)* additional copies including, where applicable, the copy for the purposes of international search under Rule 13*ter* :	
(iii) ☐	together with relevant statement as to the identity of the copy or copies with the sequence listings mentioned in left column :	
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11. ☐	other (specify) ..... :	

Figure of the drawings which should accompany the abstract: 2c

Language of filing of the international application: English

Box No. X SINGATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Isler & Pedrazzini AG

Dr. Tobias Bremi

For receiving office use only

1. Date of actual receipt of the purported international application:	23. Dez. 2003 (23. 12. 03)	2. Drawings ☐ received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:		☐ not received:
4. Date of timely receipt of the required corrections under PCT Article 11(2):		
5. International Searching Authority *(if two or more are competent):*	ISA/	6. ☐ Transmittal of search copy delayed until search fee is paid.

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Date of receipt of the record copy by the International Bureau:

SPECIFICATION

TITLE

Method for isolating cells from mammary secretion

TECHNICAL FIELD

The present invention relates to a method for isolating cells from a human body, as well as uses of such cells.

BACKGROUND OF THE INVENTION

Stem cells are defined as clonogenic, self-renewing progenitor cells that can generate a wide variety of more specialised cell types via the process of differentiation. Classically, it has been believed that there are two distinct types of stem cell. Embryonic stem (ES) cells are derived from the inner mass of the blastocyst, are pluripotent and thus are capable of generating into all differentiated cell types within the body. The other sub-population of stem cells are derived from ES cells and are organ- or tissue-specific. These multipotent cells, also known as adult stem cells, were believed to be able to differentiate only into tissues from their organ of origin. An example of these multipotent cells are haematopoietic stem cells, which serve to continually regenerate the cells of the blood and immune system.

Stem cells have been isolated from a wide range of tissues, from those that have a high rate of ongoing cellular turnover, such as blood, cord blood, bone marrow, skin, intestine, and breast tissue, to those with a low turnover such as brain, skeletal muscle,

and juvenile teeth. Irrespective of the tissue of origin, a long standing dogma has been that adult stem cells can only differentiate into the tissues from which they were derived. However recent work has demonstrated that upon exposure to a novel environment, organ-specific stem cells can overcome these intrinsic restrictions to transdifferentiate into other tissues. For example it has been shown that neural stem cells can transdifferentiate into blood cells, bone-marrow derived stem cells can transdifferentiate into muscle, brain, liver and heart cells, and skin derived stem cells can transdifferentiate into brain cells. Therefore it now appears likely that the dogma associated with developmental restriction of organ-specific stem cells is incorrect and it is feasible that these ES cells under appropriate environmental stimuli, can transdifferentiate into another cell type.

Human milk contains a mixture of different cell types. Secretory epithelial cells (lactocytes) are found in milk due to them sloughing off the basement membrane of the breast as a consequence of the pressure associated with the continued filling and emptying of the breast. Lactocytes account for approximately 10-20% of the total cell population. The majority of the remainder of cells found in human milk are leukocytes (immune cells such as lymphocytes, macrophages, monocytes, natural killer cells, basophils, eosinophils, and neutrophils), and are believed to be in milk to both protect the breast from infection and to provide immune protection for the infant. To date, these are the only cell types believed to be contained in milk.

SUMMARY OF THE INVENTION

The objective problem underlying the present invention is therefore to provide a new method for isolating progenitor cells from the human body. In this context, the term progenitor cells shall include all cells with stem cell-like characteristics, preferentially but not exclusively including pluripotent or multipotent progenitor cells like for example stem cells.

The present invention solves the above problem by deriving such cells directly or indirectly from human mammary secretion, be it colostrum, mature milk, or dry period

secretion from males or females, of said human body during at least one of the following periods: non-pregnant period, pregnant period, lactating period, involuting period. In other words, here we demonstrate that surprisingly, progenitor cells can also be found in human lactation milk and that these cells have the potential to be utilised for the generation of tissues for the mother and infant. It has to be noted that not only human mammary secretion but generally mammary secretion from mammalian species can be used for the isolation of corresponding progenitor cells. It can unambiguously be shown by means of progenitor-cell-specific antibodies, that indeed mammalian secretion, i.e. for example human milk, comprises progenitor cells.

In a first preferred embodiment of the present invention, said progenitor cells are isolated from the mammary secretion in that non-cellular parts of the mammary secretion are separated from the cellular parts and that in particular non-pluripotent or non-multipotent cells are removed from cellular parts thus derived. The cellular parts of mammary secretion, apart from pluripotent cells may further comprise secretory epithelial cells, leucocytes and in particular nonhuman cells like bacterial cells. Those non-pluripotent cells are preferentially removed from the mammary secretion.

According to another preferred embodiment of the present invention, the mammary secretion during lactating periods is used for the isolation of the progenitor cells, wherein the mammary secretion during particular stages of mammary secretion such as: after beginning of individual feeding; versus end of individual feeding; lactation phase; preferably early lactation, is used.

A particularly useful and practicable way of isolating those progenitor cells from the secretion is possible if magnet beads are used. Those magnetic beads are to this end preferentially connected to progenitor-cell-specific antibodies allowing the attachment of the beads to the progenitor cells.

Typically, in a first step cellular components are washed out of the mammary secretion, in a second step said cellular components are stained with antibodies to the progenitor cell markers, and in a third step the progenitor cells are separated from the other cells directly or indirectly by means of the attached antibodies, preferentially, but not

exclusively, by using the above-mentioned magnetic beads. To this end, the antibody-stained progenitor cells are attached to beads, preferably small iron beads, and the progenitor cells are extracted by means of the beads, preferably in case of small iron beads by using a magnet, and wherein subsequently the beads as well as if need be the antibodies are removed from the progenitor cells. This is for example possible by selecting the beads, which have been provided with specific antibodies attached to the beads, which antibodies selectively bind to the progenitor cells. To obtain the pure cells, subsequently the beads are removed from the progenitor cells, which is for example possible by means of enzymes cleaving the link between the beads and the antibodies. If the link between the beads and the antibodies is based on DNA, such cleavage can be effected by using Dnase, in case where the link between the beads and the antibodies is based on amino acid chains, proteinases can be used.

Surprisingly, while normally progenitor cells have to be cultured , i.e. grown, on very specific feeder layers, like for example mouse fibroblast feeder layers, the progenitor cells isolated in the present method do not need such specific feeder layers, but can generally be grown on other feeder layers based on for example the ones disclosed within the scope of the specific examples.

More specifically, the method of isolation comprises the following steps: (i) the whole human mammary secretion is subjected to centrifugation generally leaving a fat layer on top, a protein and carbohydrate rich supernatant beneath it, and at the bottom a pellet of cells; (ii) fat fraction and supernatant are removed; (iii) e.g. a buffer, such as, but not limited to, phosphate buffered saline, tris buffer saline, TBS and/or PBS, or media, such as, but not limited to, Williams media or RPMI Media, is added and the cells (not only comprising progenitor cells) are resuspended in the buffer / media and centrifuged as before, preferentially repeating this process 3 or 4 times, leaving a substantially pure cell pellet; (iv) the progenitor cells are separated from the cell pellet.

Preferentially, the separation of the progenitor cells from the cell pellet is carried out in the following steps:(v) the cell pellet is suspended in media, preferentially in RPMI media containing for example fetal calf (bovine) serum; (vi) this suspension is incubated

with (magnetic) beads which have preferentially before been incubated with progenitor-specific antibodies (preferentially stem cell-specific antibodies, like anti-mouse IgG antibodies), which antibodies are attached to the magnetic beads via a small strand for example of DNA or amino acids, wherein the incubation of the cell suspension in these magnetic beads is preferentially carried our for 15 minutes at 4°C; (vii) once the progenitor cells have bound to the magnetic beads a magnet is attached to the tube containing the cells/beads, thus attracting the progenitor cells connected with the beads to the magnet, whereas unbound cells are not and remain in the supernatant; (viii) removing the supernatant leaving only the progenitor cells bound to the beads via the progenitor cell antibody.

It has to be pointed out that other types of beads or generally separation means can be used which allow to selectively attach them to the progenitor cells. Such beads have to be separated from the progenitor cells, and to this end preferentially the following processing steps can be used: (ix) progenitor cells bound to the beads via the stem cell antibody are removed by an appropriate cleavage means, preferentially, in case of the antibody being attached to the beads via small strand of DNA, a by means of addition of a Dnase, (x) the beads are removed by attaching the magnet once more, such that the beads, no longer attached to the stem cells, are attracted to it; (xi) removing the supernatant now containing the isolated progenitor cells.

The present invention furthermore relates to progenitor cells, which are preferentially pluripotent or multipotent progenitor (stem) cells, derived using a method as described above.

Additionally, the present invention relates to uses of such progenitor cells, for example for ex vivo, in vitro and/or in vivo applications. Without limiting the scope of the invention, such use may extend to the following specific examples or combinations thereof : creation of tissues or cells for the benefit of the mother and/or of the infant and/or of other individuals; subsequent gene therapy treatments or intrauterine foetal treatments; generation of cells, tissue, glands or organs for the treatment of disease; subsequent cloning or scientific research; one or several of the group of the following

purposes: clinical, diagnostic, bioengineering, lactoengineering, breast tissue regeneration, breast reconstructive surgery, breast cosmetic or enhancement surgery, exocrine gland tissue regeneration and/or surgery.

Further embodiments of the present invention are outlined in the dependent claims.

SHORT DESCRIPTION OF THE FIGURES

In the accompanying drawings preferred embodiments of the invention are shown in which:

Figure 1 shows the total mixed cell population of human milk following removal of the fat and skim milk;

Figure 2 shows the stem cells (sc) following their isolation from a total milk cell population. These cells have been cytospun onto microscope slides and stained with haematoxylin and eosin. Figure 2a) and 2b) show a single stem cell (sc). Figure 2c) shows a single stem cell still bound to the Dynabeads® (Db). The Dynabeads® (Db) are 4.5μM in size, thereby permitting the approximate sizing of the stem cells to be 6-7μM;

Figure 3 following purification of the stem cells from a total milk cell population, the stem cells that are isolated can be cultured using a variety of culture conditions; this figure shows a single stem cell 1 day after being placed into culture;

Figure 4 after 1 to 2 weeks in culture, the stem cells isolated from a total cell population begin to undergo cell division (mitosis); the arrow in this photo indicates a cell undergoing mitosis;

Figure 5 after several months in culture, these cells do not appear to have differentiated into other cell types; this figure shows a stem cell after 2 months of culture which has been cytospun onto a microscope slide and stained with haematoxylin and eosin;

Figure 6 after 2 months in culture the stem cells have not differentiated; this figure shows the stem cells isolated with the SSEA-4 (a) and Tra 1-60 (b) antibodies, following 2 months in culture; the cells were washed out of the culture medium and cytospun onto a microscope slide before being visualised on a confocal microscope with the SSEA-4 (a) and Tra 1-60 (b) antibodies conjugated to a fluorescently labelled secondary antibody (goat anti-mouse IgG conjugated to AlexaFluor-488).

Figure 7 shows the growth rate of isolated stem cells in culture; cells were seen to divide and proliferate over a period of 4 days; cells isolated with Dynabeads® and antibody SSEA-4 have shown an increase in total number of cells from approximately 50 cells per plate to 150 cells per plate in 4 days of culture; the central area of the plates was determined and a cruciform counting pattern was used; N=10 fields in each culture and numbers on individual days were simply totalled; the data shown in this Figure is the same experiment performed in triplicate

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

To determine whether a cell believed to be a stem cell is actually a stem cell, it has historically been necessary to undertake transplantation of the cells of interest into sub-lethally irradiated mice. If these transplanted cells subsequently locate and repopulate any organs of these mice, the cells in question have been considered to be pluripotent stem cells. However, more recently the identification of extracellular markers for pluripotent stem cells (for example Tra-1-60 and SSEA-4; Chemicon International, Temecula, California, US) have enabled the identification of pluripotent (stem) cells without the prolonged process of transplantation.

After washing all cells out of human milk by a repeating the process of gentle centrifugation, aspiration of the supernatant and the resupension of the cells in a buffer or media several times, the stem cells were isolated from a total milk cell population (Figure 1) using dynabeads according to manufacturers specifications. As buffer Tris-

buffered saline solutions (TBS) or phosphate buffered saline solutions (PBS) can be used. In particular, TBS-solutions which are 10 mM in Tris, 150 mM in NaOH and which are adjusted to have a pH around 7.4 can be used. In case of PBS-solutions, those may be 1.1mM KH_2PO_4, 140mM NaCl, 4.5mM $Na_2HPO_4.7H_2O$ and 2.7mM NaCl, and may be adjusted to have a pH around 7.4. As media, Williams or RPMI (Roswell Park Memorial Institute) as available from Sigma Aldrich, US, under the product numbers R6504, R7755, R4130 or W4125, W4128, W1878, can be used. Also possible as media is RPMI from Gibco (CA, US) Cat. No 11875-093.

Stem cell-specific antibodies, e.g. Tra-1-60, Tra 1-81 and/or SSEA-4, both monoclonal, both e.g. available from Chemicon international, CHEMICON International, 28820 Single Oak Drive, Temecula CA 92590, were attached to the Dynabeads® for isolation of the progenitor cells. Also possible is the use of haematopoietic cells antibody CD133 (cat# MAB1133) from R&D Systems, Inc CA, US.

Primitive haematopoietic stem and progenitor cells from peripheral blood have already been isolated using the immuno-magnetic cell separation principles. Research groups using monoclonal-antibody conjugated magnetic particles (CliniMACS System and Reagent AC133 from Miltenyi Biotec) have successfully isolated and cultured CD133 positive cells.

Any stem cell-specific antibody (inclusive of; SSEA-3, SSEA-1 and TRA 1-81 Oct-4, CD133) are incubated with Dynabeads®. Dynabeads® are available from Dynal AS, NO, and are small iron beads, that have an anti-mouse IgG antibody attached to it via a small strand of DNA. Possible are for example Dynabeads® available under the name Dynal CD34. Incubation of the Dynabeads® is carried out for 1h at room temperature prior to the Dynabeads® being incubated with the cells isolated from milk for 15 minutes at 4°C.

Once the stem cells have bound to the Dynabeads which typically takes about 30 minutes to 1 hour, a magnet is attached to the side of the tube containing the cells/Dynabeads®. Dynabeads® are uniform, polystyrene-based, paramagnetic, beads with 4.5 µm in diameter. The Dynabeads® (with the stem cells attached) are attracted to the magnet, whereas the unbound cells are not and remain in the supernatant. The

supernatant is then removed leaving only the cells bound to the Dynabeads® via the stem cell antibody. The cells bound to the Dynabeads® via the stem cell antibody are removed by the addition of Dnase breaking the small strand of DNA. This is called releasing buffer and is part of the Dynal kit 62500 U/ml (15000-20000U per vial quoted in the instructions). The Dynabeads® are removed by attaching the magnet once more to which the Dynabeads®, no longer attached to the stem cells, are attracted. The supernatant now contains the stem cells which are removed. These stem cells can now be used for any subsequent application as listed above/below.

The isolated progenitor (stem) cells can be cultured on mouse embryo fibroblast feeder cells in Knockout-Dulbecco's modified Eagle's medium. Typically cultivation can be carried out at a temperature of 37°C.

Examples of the use of feeder cells:

- Prolonged propagation of human embryonic stem cells is currently achieved by coculture with primary mouse embryonic fibroblasts serving as feeder cells.
- Acceleration of the formation of cultured epithelium using the sonic hedgehog expressing feeder cells.
- Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture.
- Proliferation and maturation of embryonic stem cells into endothelial cells in an in vitro model of vasculogenesis required OP9 feeder cells.
- Selective expansion and continuous culture of macrophages from adult pig blood. Macrophages were selectively expanded and continuously cultured from adult pig blood directly into the medium overlaying a feeder layer of STO mouse fibroblasts.
- Establishment and characterization of a novel human immature megakaryoblastic leukemia cell line, M-MOK, dependent on fibroblasts for its viability. A novel fibroblast-dependent human immature megakaryoblastic leukemia cell line (M-MOK) was established from the bone marrow of a girl

with acute megakaryoblastic leukemia, and its growth was determined to be completely dependent on the presence of human embryonic lung-derived fibroblasts, HEL-O.

- In vitro culture of embryonic disc cells from porcine blastocysts using foetal G30 porcine fibroblasts which had been previously irradiated as a feeder layer.
- Acute lymphocytic leukemias (ALL) cells of infants and children were found to preferentially survive in coculture with a cloned cell line of endothelial adipose cells (14F1.1) from mouse bone marrow and exhibited extensive growth in the presence of the mouse stromal cells. These ALL cells were strictly dependent upon the mouse stromal clone 14F1.1 and failed to proliferate in the absence of the endothelial adipocytes or with a variety of feeder cells.

Cell growth

Cells isolated with Dynabeads® and antibody SSEA-4 have shown an increase in total number of cells from approximately 50 cells per plate to 150 cells per plate in 4 days (see Figure 7).

Methods:

Cell preparation

150 ml of whole milk is spun for 15 minutes at 2000rpm to collect cells. Cell pellet is resuspended in approximately 4ml TBS 1% BSA and centrifuged again for 10 minutes.

Final pellet is resuspended in 200µl of RPMI 1% FCS.

Bead preparation

Beads are washed 3x in RPMI 1% foetal calf serum.

Beads coated with primary antibody at a concentration of 1ul in 500 µl TBS 1% BSA and incubated for 1 hour at room temperature with gentle mixing.

Coated beads separated and washed 3x in 1ml TBS 1% BSA and transferred to clean tube. Final wash in 1ml RPMI 1% FCS.

Cell isolation

200 µl cell preparation added to beads and incubated for 30min at approximately 100°C.

Following incubation the unbound fraction is discarded.

200 µl RPMI 1% FCS is added to the cell and bead complex which is then gently pipetted to release further unbound matter.

To 200 µl of bead and cell complex add 5 µl releasing buffer and incubate for 15 minutes at 37°C with gentle mixing.

Vigorously pipette bead plus cell complex to help release cells.

Collect the 200 µl of isolated cell suspension and add to clean Eppendorf.

This cell suspension can then be immediately introduced into the culture system.

Culture Method

Cells were seeded on collagen-coated culture plates.

Following a settling period the supernatant was carefully drawn off to remove contaminants and fresh media added. Cultures were then incubated in plates at 37°C in a 5% CO_2 incubator with medium changes every two days.

Culture media

William's E medium supplemented with 10% fetal calf serum; 10^{-7}M dexamethasone (Sigma); Glutamine 2mmol/L ITS+ premix containing insulin (6.25µg/ml), transferrin (6.25µg/ml), selenious acid (6.25ng/ml), bovine serum albumin (1.25mg/ml) and linoleic acid (5.35µg/ml) from Becton-Dickinson, Bedford, MA. Penicillin / streptomycin 5,000 µg/ml Fungizone (250µg/ml)

Antibodies

An example of some of the potential antibodies that can be used in this system are; (All supplied by CHEMICON International, 28820 Single Oak Drive, Temecula CA 92590)

SSEA-1 (Cat number MAB4301)

SSEA-3 (MAB4303)

SSEA-4 (MAB4304)

TRA 1-60 (MAB4360)

TRA 1-81 (MAB4381)

haematopoietic cells antibody CD133 (cat# MAB1133) from R&D Systems, Inc.

Buffers

TBS 1% BSA, 10mM Tris, 150 mM NaOH

RPMI 1% FCS

PBS 1.1 mM KH_2PO_4, 140 mM NaCl, 4.5 mM Na_2HPO_4 $7H_2O$

Following purification of the stem cells from human milk, using the extracellular markers for stem cells (Tra-1-60 and SSEA-4) bound to Dynabeads® we cytospun the stem cells onto microscope slides and stained the stem cells using haematoxylin and eosin (Figure 2). Using these same purified stem cells we have been able to culture the cells (Figure 3) and demonstrate that these cells do undergo cell division (Figure 4). Following prolonged culture, we have been able to stain these cells using haematoxylin and eosin (Figure 5), and have demonstrated that these cells remain stem cells as they continue to bind the stem cell antibodies (Tra-1-60 and SSEA-4) following several months of culture, as visualised by confocal microscopy (Figure 6). Growth of the isolated cells was also verified, see Figure 7.

Due to the plasticity of the pluripotent stem cells, these isolated cells can be utilised for a multitude of different applications. For example these cells can be;

- used to create tissues for the benefit of the mother and infant (and potentially other individuals), including gene therapy treatments, intrauterine foetal treatments, and the generation of cells, tissue, glands, or organs for the treatment of disease. This includes their use in scientific research, clinical, diagnostic or commercial applications. This can also include the generation of biological compounds including cells, cellular compartments, cellular secretions, cell isolates, nucleotides, deoxyribonucleic acids, amino acids, proteins, glycoproteins, carbohydrates, lipids, hormones, growth factors, and cytokines. Furthermore, this can include the generation of cells as a precursor, or as a consequence, of the generation of tissue, glands or organs for the treatment of disease, tissue regeneration, body enhancement, or cosmetic applications for the following tissues; Olfactory, Auditory, Optical, Lymphatic, Immune, Haematopoietic, Endocrine, Exocrine, Bowel, Gastrointestinal, Peyers Patches, Islets of Langerhans, Skeletal, Muscle, Connective, Vascular, Blood, Skin, Hair, Nails, Mammary, Brain and Central Nervous System, Liver, Heart, Lung, Kidney, Bone, Pancreas, Reproductive.

- stored for future. The subsequent use of these stem cells, or cells differentiated or dedifferentiated could include storage of these stem cells for future use as outlined below. This includes their storage for use in scientific research, clinical, diagnostic or commercial applications.

- used for cell culture, whether this be for propagation of these same stem cells, or for the differentiation or dedifferentiation of these stem cells into another cell type. This includes their use in scientific research, clinical, diagnostic or commercial applications.

- used for cloning. The subsequent use of these stem cells, or cells differentiated or dedifferentiated from these stem cells could be used to generate clones, whether embryonic or whole animal. This includes their use in scientific

research, clinical, diagnostic or commercial applications.

- used for scientific research. The subsequent use of these stem cells, or cells differentiated or dedifferentiated from these stem cells could be used in scientific research. This can include the generation of biological compounds including cells, cellular compartments, cellular secretions, cell isolates, nucleotides, deoxyribonucleic acids, amino acids, proteins, glycoproteins, carbohydrates, lipids, hormones, growth factors, and cytokines. In addition this could include the generation of cells as a precursor, or as a consequence, of the generation of tissue, glands or organs for the treatment of disease, tissue regeneration, body enhancement, or cosmetic applications for the following tissues; Olfactory, Auditory, Optical, Lymphatic, Immune, Haematopoietic, Endocrine, Exocrine, Bowel, Gastrointestinal, Peyers Patches, Islets of Langerhans, Skeletal, Muscle, Connective, Vascular, Blood, Skin, Hair, Nails, Mammary, Brain and Central Nervous System, Liver, Heart, Lung, Kidney, Bone, Pancreas, Reproductive.

- used for clinical, diagnostic or commercial applications. The subsequent use of these stem cells, or cells differentiated or dedifferentiated from these stem cells could be used in clinical, diagnostic or commercial applications. This can include the generation of biological compounds including cells, cellular compartments, cellular secretions, cell isolates, nucleotides, deoxyribonucleic acids, amino acids, proteins, glycoproteins, carbohydrates, lipids, hormones, growth factors, and cytokines. In addition this could include the generation of cells as a precursor, or as a consequence, of the generation of tissue, glands or organs for the treatment of disease, tissue regeneration, body enhancement, or cosmetic applications for the following tissues; Olfactory, Auditory, Optical, Lymphatic, Immune, Haematopoietic, Endocrine, Exocrine, Bowel, Gastrointestinal, Peyers Patches, Islets of Langerhans, Skeletal, Muscle, Connective, Vascular, Blood, Skin, Hair, Nails, Mammary, Brain and Central Nervous System, Liver, Heart, Lung, Kidney, Bone, Pancreas, Reproductive.

- used for bioengineering. The subsequent use of these stem cells, or cells

differentiated or dedifferentiated from these stem cells could be used to generate any other cell type in the human body. These cells, tissues, or organs could then be used for cosmetic/reconstructive surgery, organ/tissue transplantation or the generation of cells/tissue/organs for a third party. This can include the generation of biological compounds including cells, cellular compartments, cellular secretions, cell isolates, nucleotides, deoxyribonucleic acids, amino acids, proteins, glycoproteins, carbohydrates, lipids, hormones, growth factors, and cytokines. In addition this could include the generation of cells as a precursor, or as a consequence, of the generation of tissue, glands or organs for the treatment of disease, tissue regeneration, body enhancement, or cosmetic applications for the following tissues; Olfactory, Auditory, Optical, Lymphatic, Immune, Haematopoietic, Endocrine, Exocrine, Bowel, Gastrointestinal, Peyers Patches, Islets of Langerhans, Skeletal, Muscle, Connective, Vascular, Blood, Skin, Hair, Nails, Mammary, Brain and Central Nervous System, Liver, Heart, Lung, Kidney, Bone, Pancreas, Reproductive.

- used for lactoengineering. The subsequent use of these stem cells, or cells differentiated or dedifferentiated from these stem cells could be used to generate biological compounds of milk including cells, cellular compartments, cellular secretions, cell isolates, nucleotides, deoxyribonucleic acids, amino acids, proteins, glycoproteins, carbohydrates, lipids, hormones, growth factors, and cytokines.

- breast tissue regeneration. The subsequent use of these stem cells, or cells differentiated or dedifferentiated from these stem cells could be used to generate breast tissue.

- breast reconstructive surgery. This regenerated tissue as above could then be used for reconstructive breast surgery.

- breast cosmetic surgery This regenerated tissue as above could then be used for cosmetic breast surgery.

- exocrine gland tissue regeneration and/or surgery. The subsequent use of these

stem cells, or cells differentiated or dedifferentiated from these stem cells could be used to generate exocrine gland tissue, which in turn, could be used for the regeneration or replacement of exocrine glands.

- the generation of biological compounds including cells, cellular compartments, cellular secretions, cell isolates, nucleotides, deoxyribonucleic acids, amino acids, proteins, glycoproteins, carbohydrates, lipids, hormones, growth factors, and cytokines.

CLAIMS

1. A method for isolating progenitor cells from a human body, inclusive of all cells with stem cell-like characteristics, wherein such cells are directly or indirectly derived from human mammary secretion, be it colostrum, mature milk, or dry period secretion from males or females, of said human body during at least one of the following periods: non-pregnant period, pregnant period, lactating period, involuting period.

2. A method according to claim 1, wherein the progenitor cells are pluripotent or multipotent.

3. A method according to claim 1, wherein said progenitor cells are isolated from the mammary secretion in that noncellular parts of the mammary secretion are separated from the cellular parts.

4. A method according to claim 3, wherein non-pluripotent or non-multipotent cells are removed from cellular parts.

5. A method according to any of the preceding claims, wherein secretory epithelial cells, leucocytes and in particular nonhuman cells like bacterial cells are removed from the mammary secretion.

6. A method according to any of the preceding claims, wherein the mammary secretion during lactating periods is used for the isolation of the progenitor cells, and wherein the mammary secretion during particular stages of mammary secretion such as: after beginning of individual feeding; versus end of individual feeding; lactation phase; preferably early lactation.

7. A method according to any of the preceding claims, wherein magnet beads are used to the isolation of the progenitor cells.

8. A method according to any of the preceding claims, wherein in a first step cellular components are washed out of the mammary secretion, in a second step said cellular components are stained with antibodies to the progenitor cell markers, and in a third step the progenitor cells are separated from the other cells directly or indirectly by means of the attached antibodies.

9. A method according to claim 8, wherein the antibody-stained progenitor cells are attached to beads, preferably small iron beads, and wherein the progenitor cells are extracted by means of the beads, preferably in case of small iron beads by using a magnet, and wherein subsequently the beads as well as if need be the antibodies are removed from the progenitor cells.

10. A method according to claim 9, wherein removal of the beads is effected by means of enzymes selected from the following group: Dnase, Proteinase, Rnase.

11. A method according to any of the preceding claims, wherein the progenitor cells are cultured without using a fibroblast feeder layer, in particular without using a mouse fibroblast feeder layer.

12. A method according to any of the preceding claims, wherein in
(i) a first step the whole human mammary secretion is subjected to centrifugation leaving a fat layer on top, a protein and carbohydrate rich supernatant beneath it, and at the bottom a pellet of cells;

(ii) in a second step fat fraction and supernatant are removed;

(iii) in a third step a buffer, such as, but not limited to, phosphate buffered saline, tris buffer saline, TBS and/or PBS, or media, such as, but not limited to, Williams media or RPMI Media, is added and the cells are resuspended in the buffer / media and centrifuged as before, preferentially repeating this process 3 or 4 times, leaving a substantially pure cell pellet;

(iv) and in a fourth step the progenitor cells are separated from the cell pellet.

13. A method according to any preceding claim, wherein a cell pellet is generated from the human mammary secretion, and subsequently the following separation steps are used:

(v) the cell pellet is suspended in media, preferentially in RPMI media containing foetal calf (bovine) serum,

(vi) this suspension is incubated with magnetic beads which have before been incubated with progenitor, preferentially stem cell-specific antibodies, like anti-mouse IgG antibodies, which antibodies are attached to the magnetic beads via a small strand of DNA, wherein the incubation of the cell suspension is preferentially carried our for 15 minutes at 4°C;

(vii) once the progenitor cells have bound to the magnetic beads a magnet is attached to the tube containing the cells/beads, thus attracting the progenitor cells connected with the beads to the magnet, whereas unbound cells are not and remain in the supernatant;

(viii) removing the supernatant leaving only the progenitor cells bound to the beads via the progenitor cell antibody.

14. A method according to claim 13, wherein subsequently, the following steps are used:

(ix) progenitor cells bound to the beads via the stem cell antibody are removed by an appropriate cleavage means, preferentially, in case of the antibody being

attached to the beads via small strand of DNA, a by means of addition of a Dnase,

(x) the beads are removed by attaching the magnet once more such that the beads, no longer attached to the stem cells, are attracted to it;

(xi) removing the supernatant now containing the isolated progenitor cells.

15. Progenitor cells, preferentially pluripotent or multipotent progenitor cells, derived using a method according to any of the preceding claims 1 through 14.

16. Use of pluripotent or multipotent progenitor cells as derived using a method according to any of the claims 1-14 for ex vivo, in vitro and/or in vivo applications.

17. Use according to claim 16, to create tissues or cells for the benefit of the mother and/or of the infant and/or of other individuals.

18. Use according to claim 16 or 17, including subsequent gene therapy treatments or intrauterine foetal treatments.

19. Use according to claim 16-18, for the generation of cells, tissue, glands or organs for the treatment of disease.

20. Use according to any of the claims 16-20, for subsequent cloning or scientific research.

21. Use according to any of the claims 16-20, for one or several of the group of the

following purposes: clinical, diagnostic, bioengineering, lactoengineering, breast tissue regeneration, breast reconstructive surgery, breast cosmetic or enhancement surgery, exocrine gland tissue regeneration and/or surgery.

5

SUMMARY

The present invention relates to a method for isolating progenitor cells from a human body, inclusive of all cells with stem cell-like characteristics, in particular pluripotent or multipotent progenitor cells, wherein such cells are directly or indirectly derived from human mammary secretion, be it colostrum, mature milk, or dry period secretion from males or females, of said human body during at least one of the following periods: non-pregnant period, pregnant period, lactating period, involuting period. The present invention furthermore relates to preferred uses of such isolated cells.

(Fig. 2c)

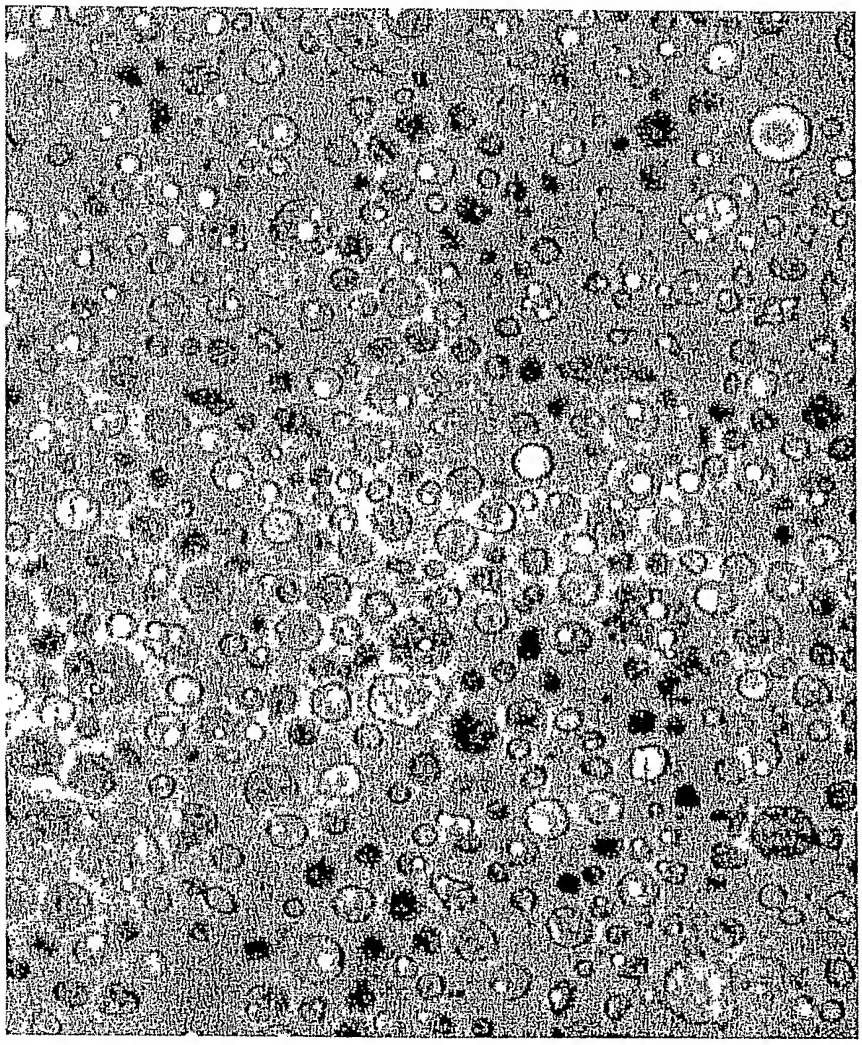
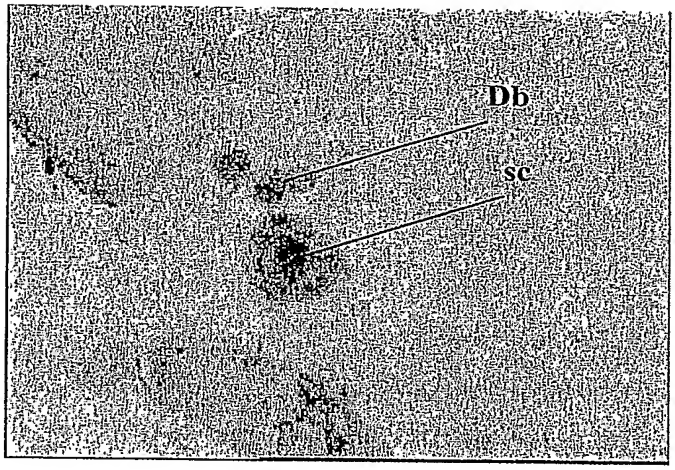


Fig. 1

a)



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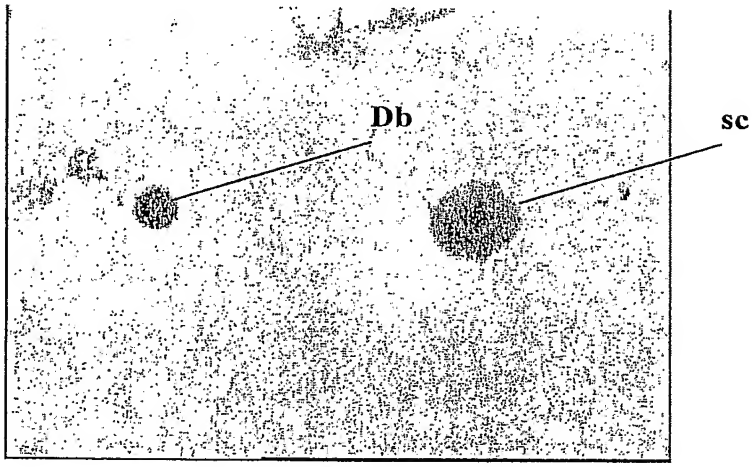

Fig. 2

c)

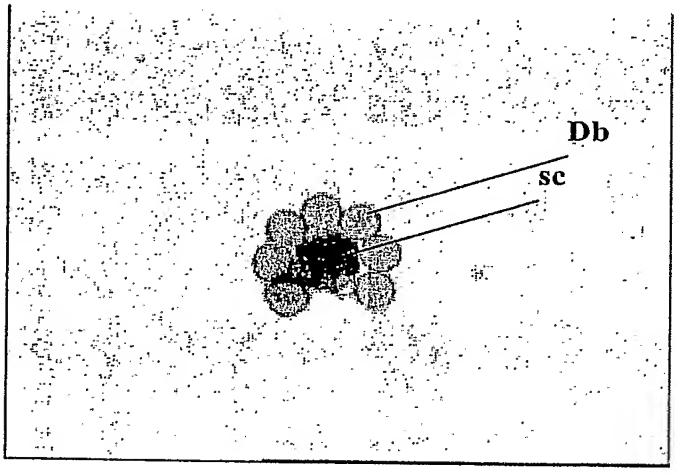

Fig. 2

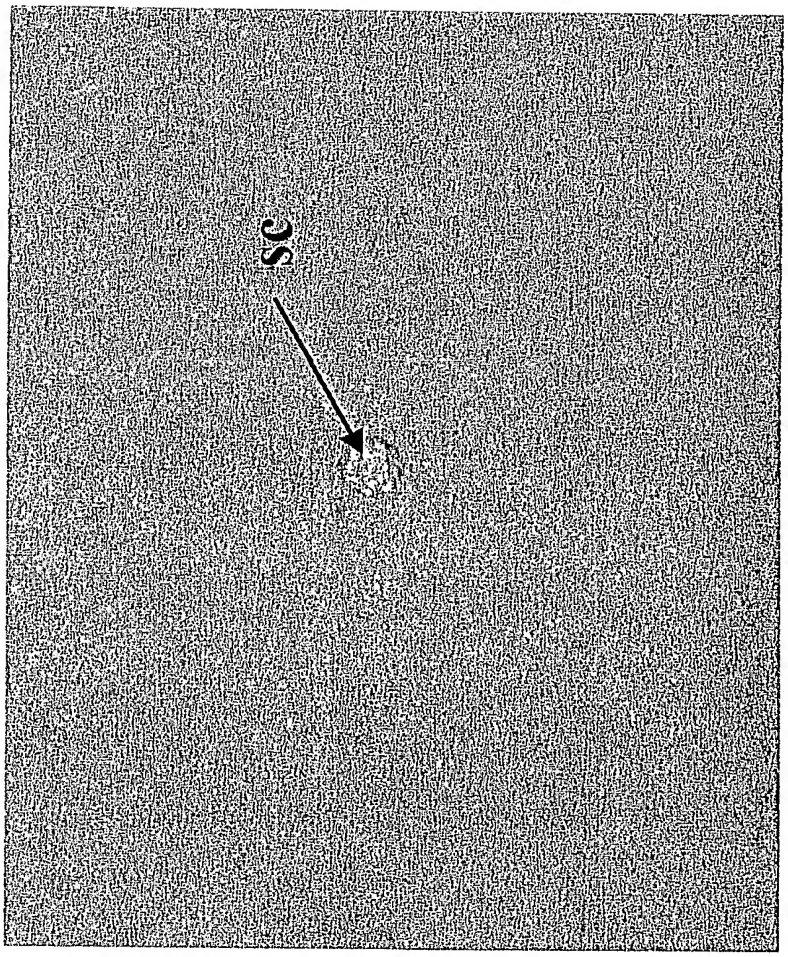

Fig. 3

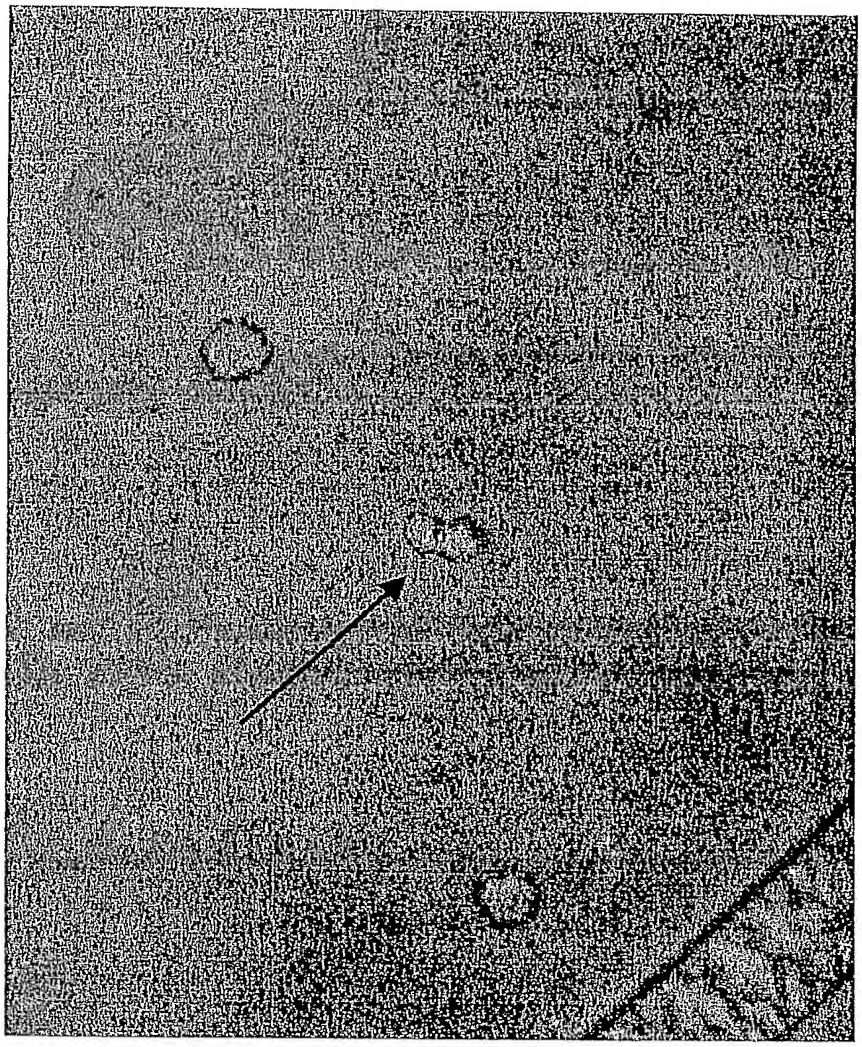

Fig. 4

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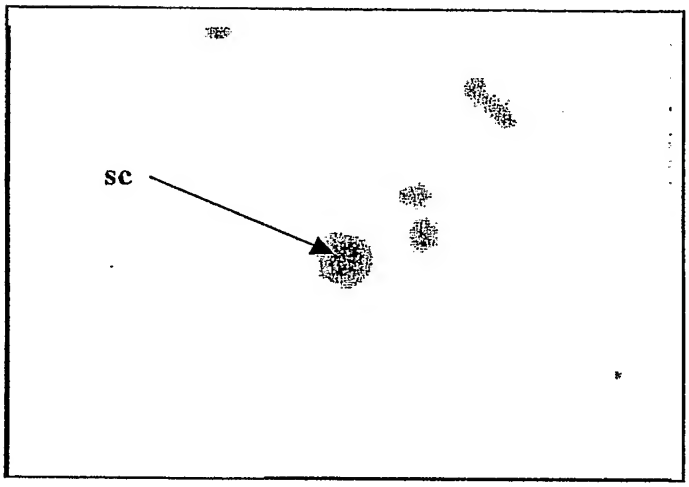


Fig. 5

5

a)

b)

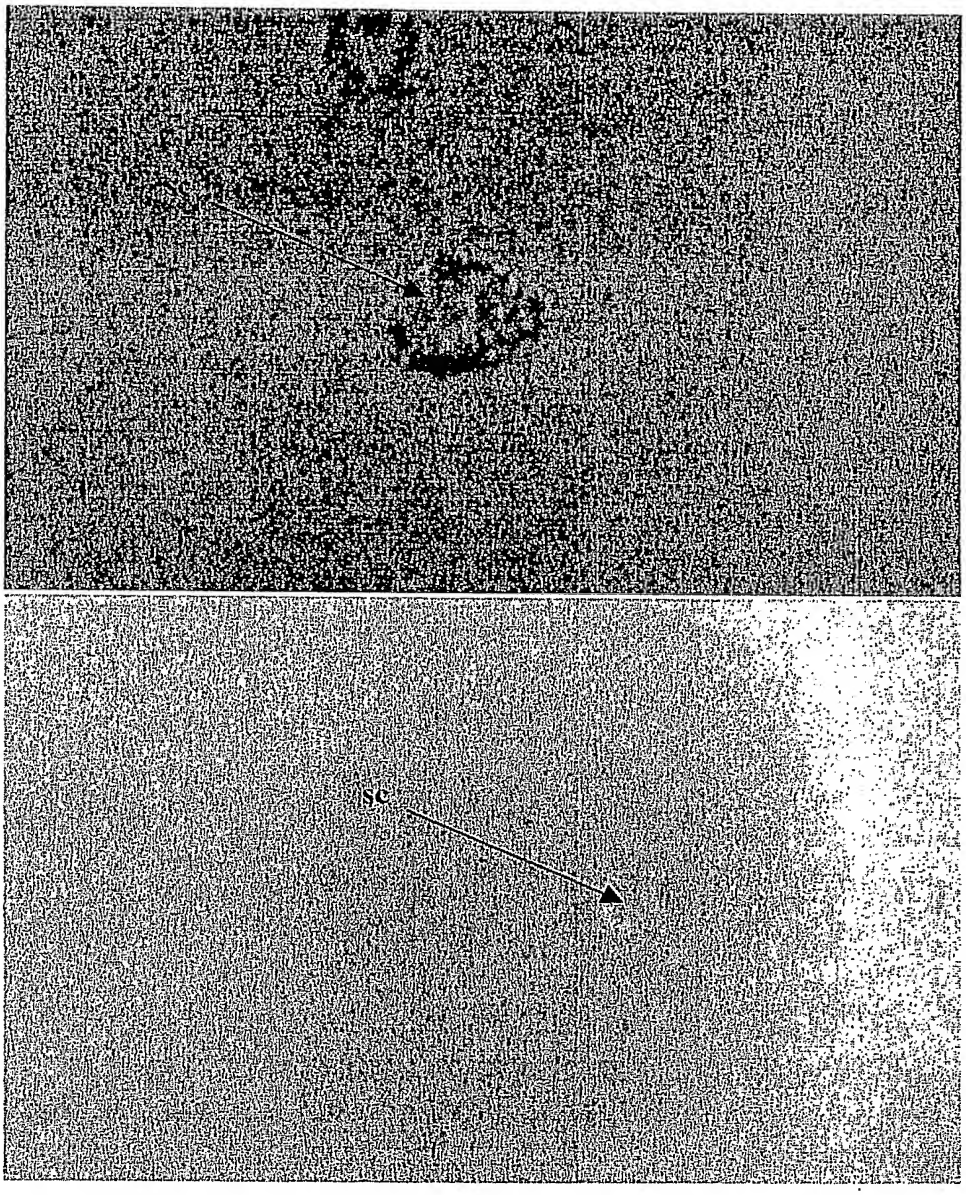


Fig. 6

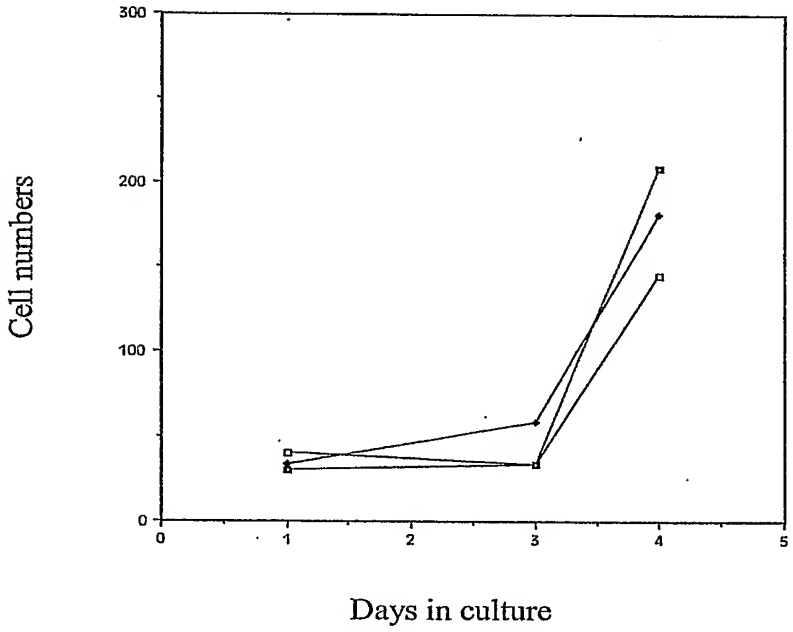


Fig. 7

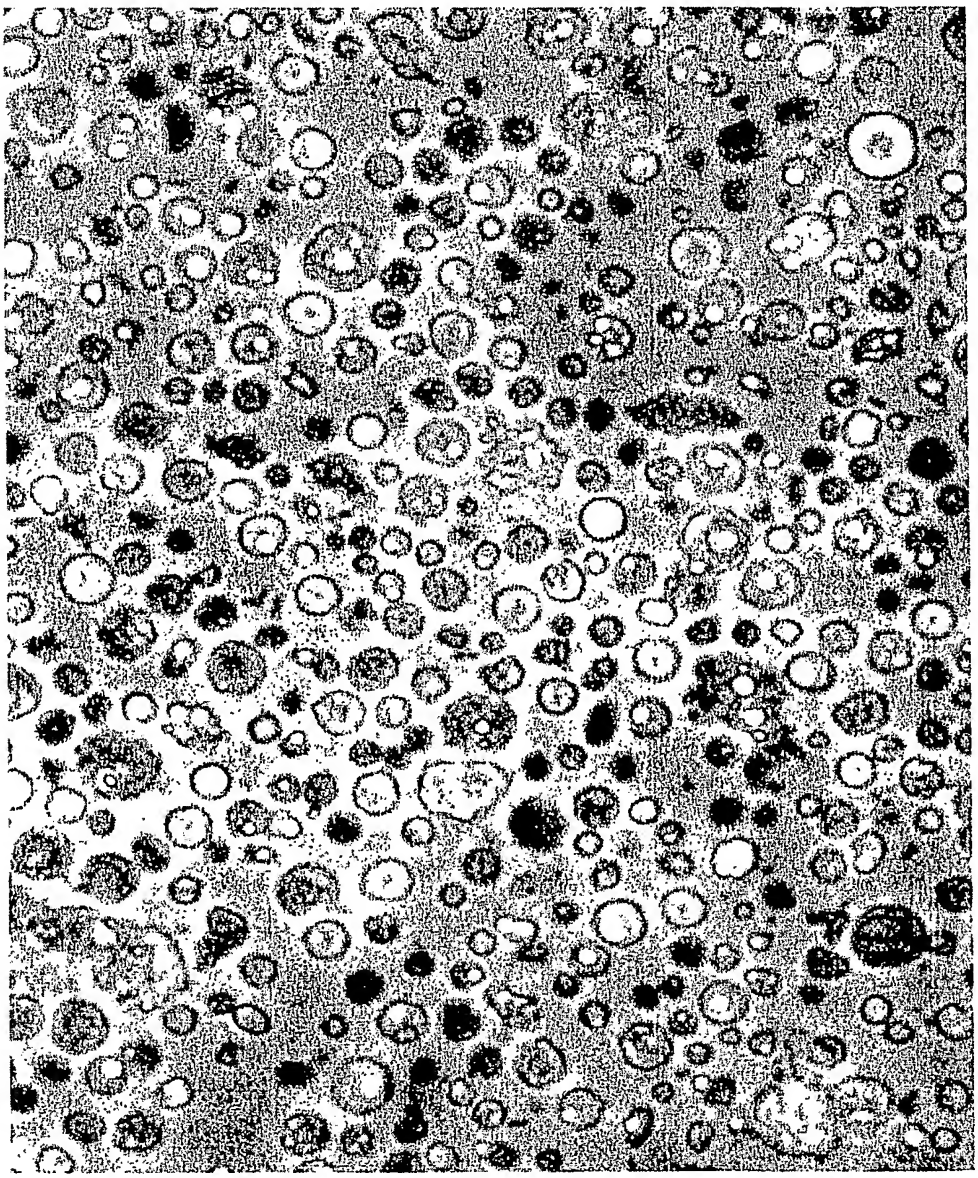
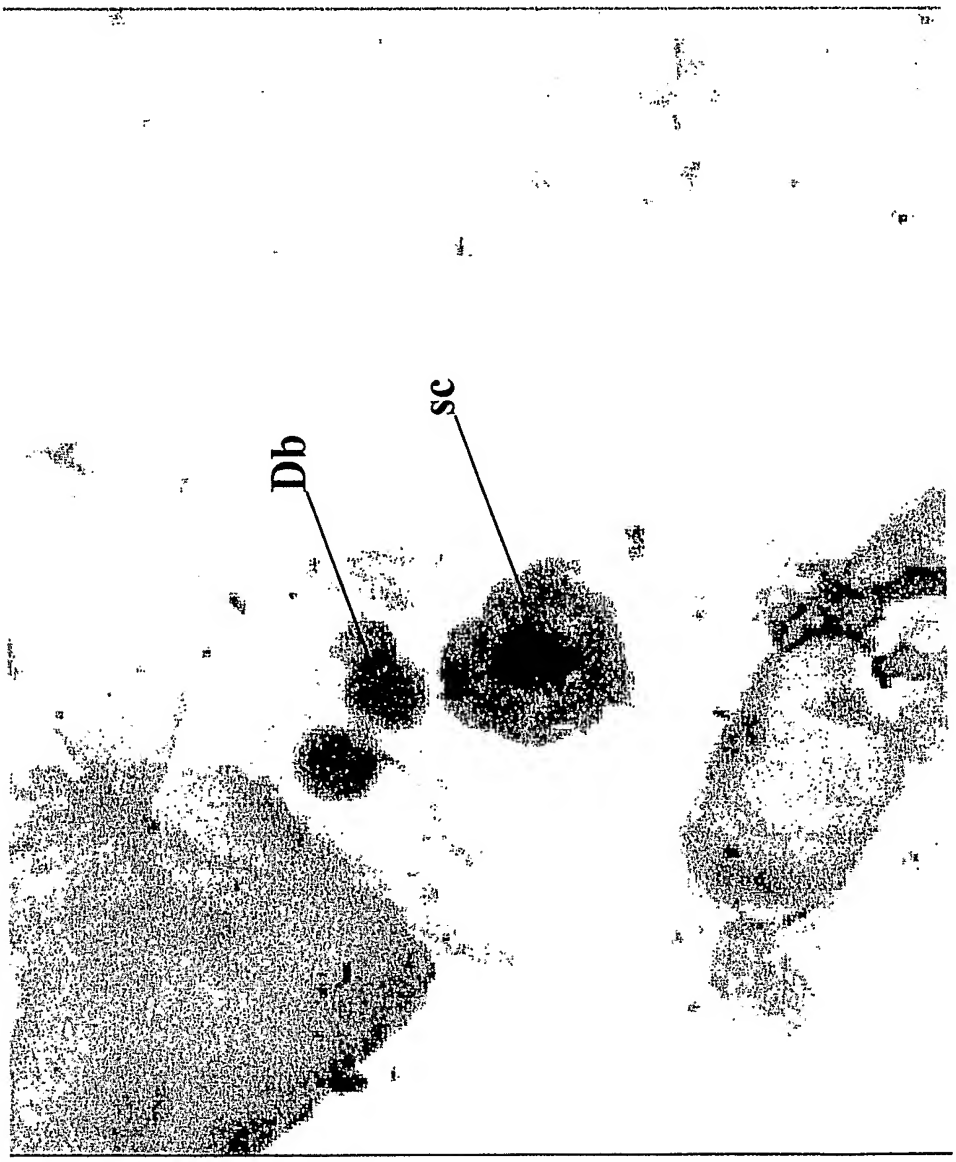
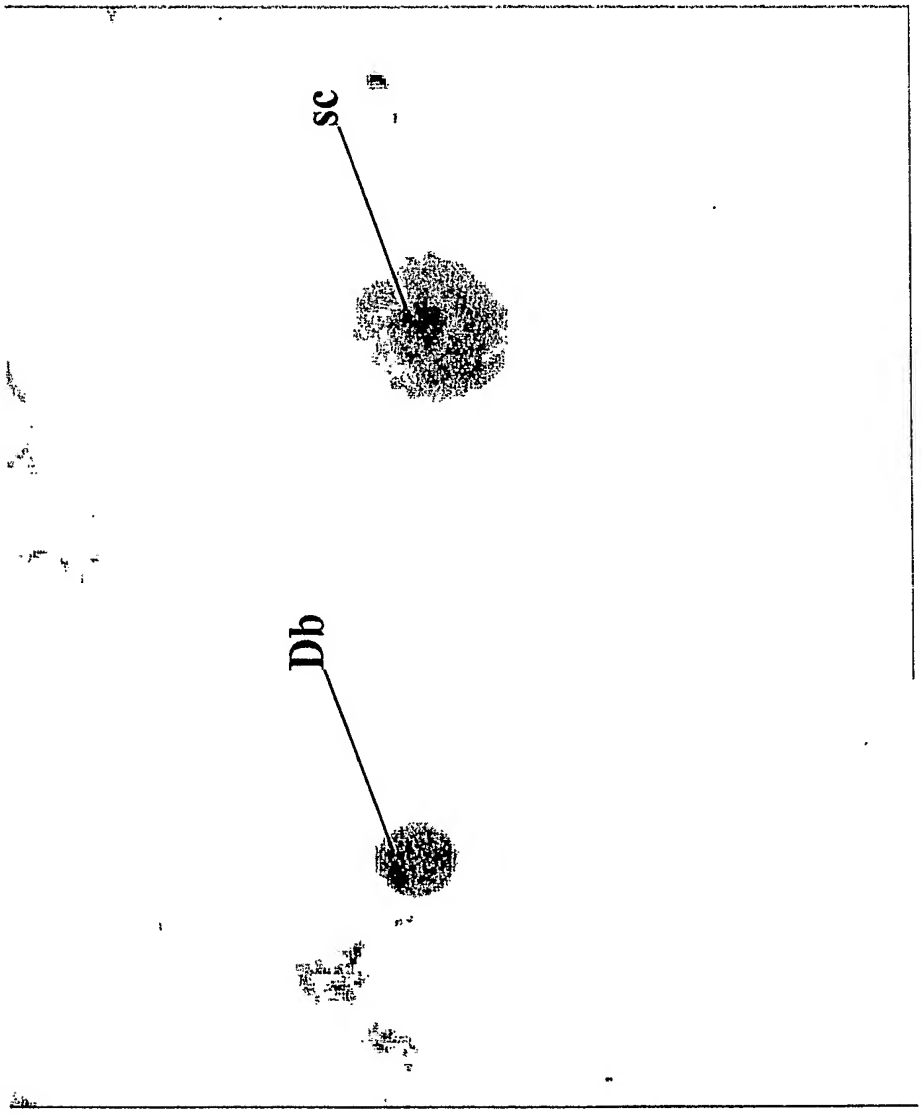


Fig. 1



a)

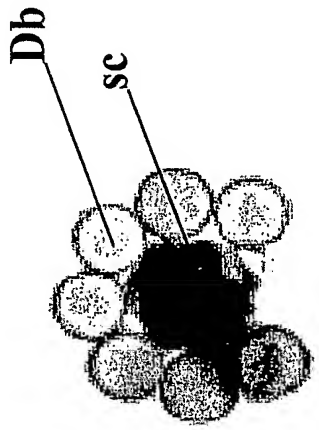
Fig. 2



b)

Fig. 2

Fig. 2



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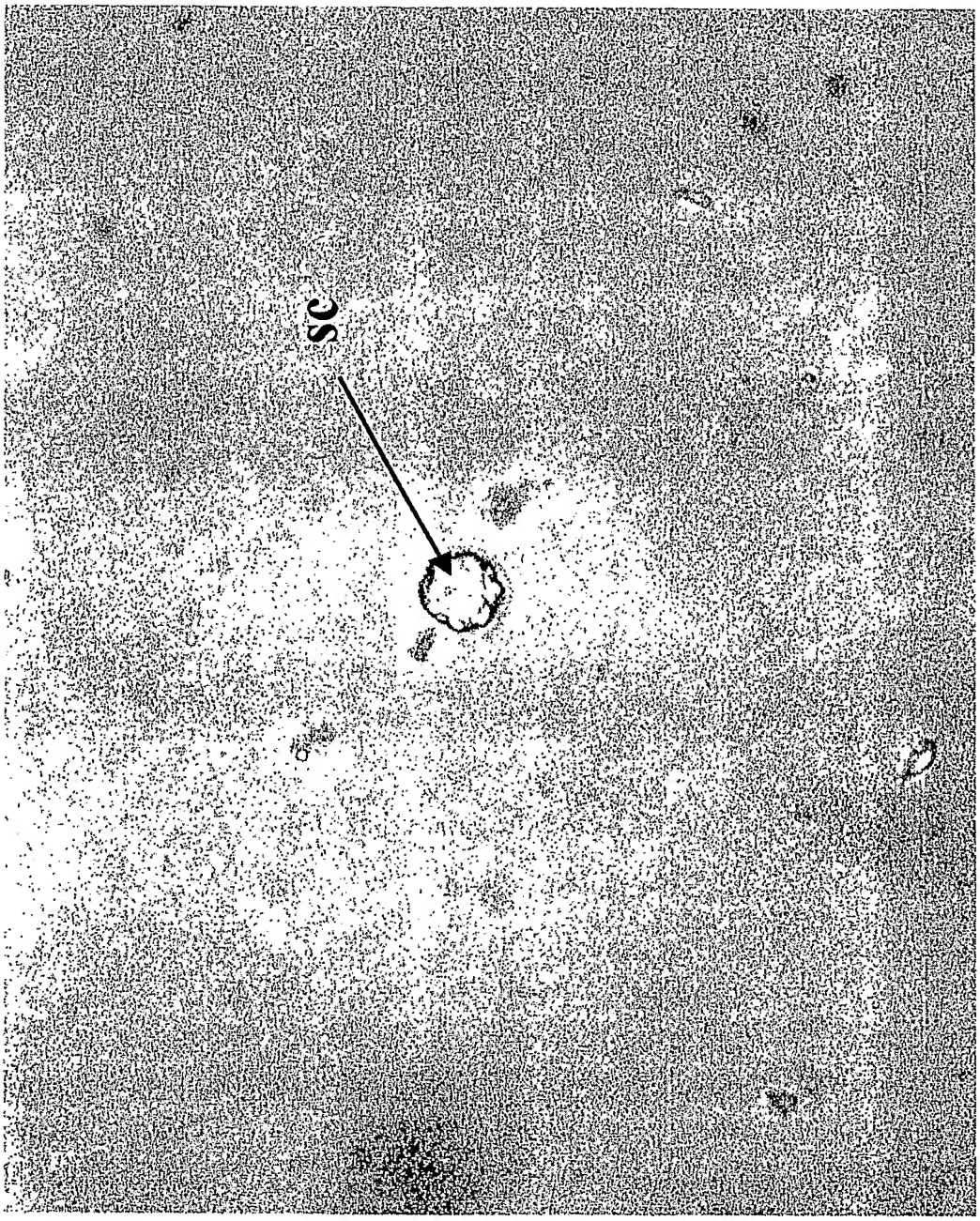


Fig. 3

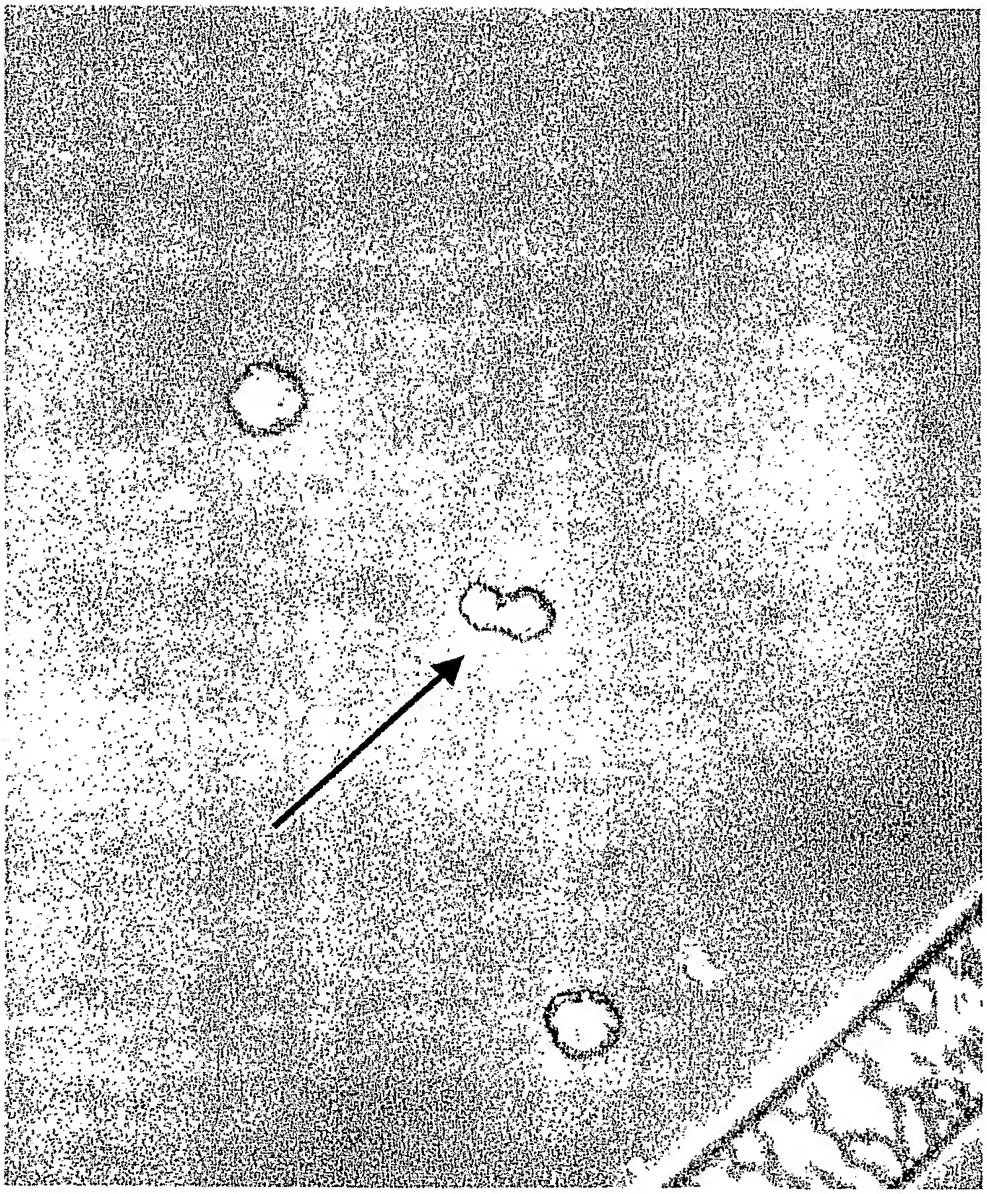


Fig. 4

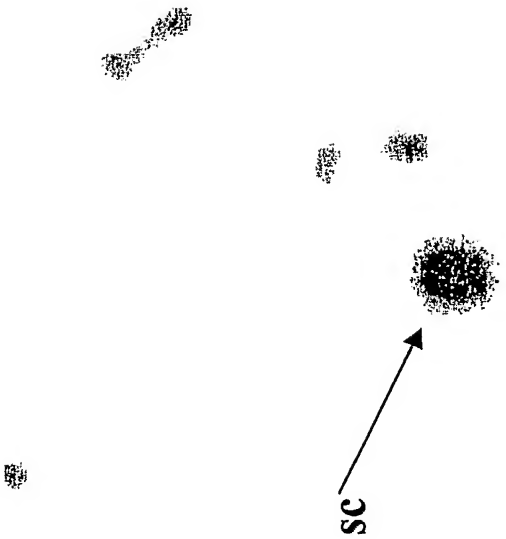
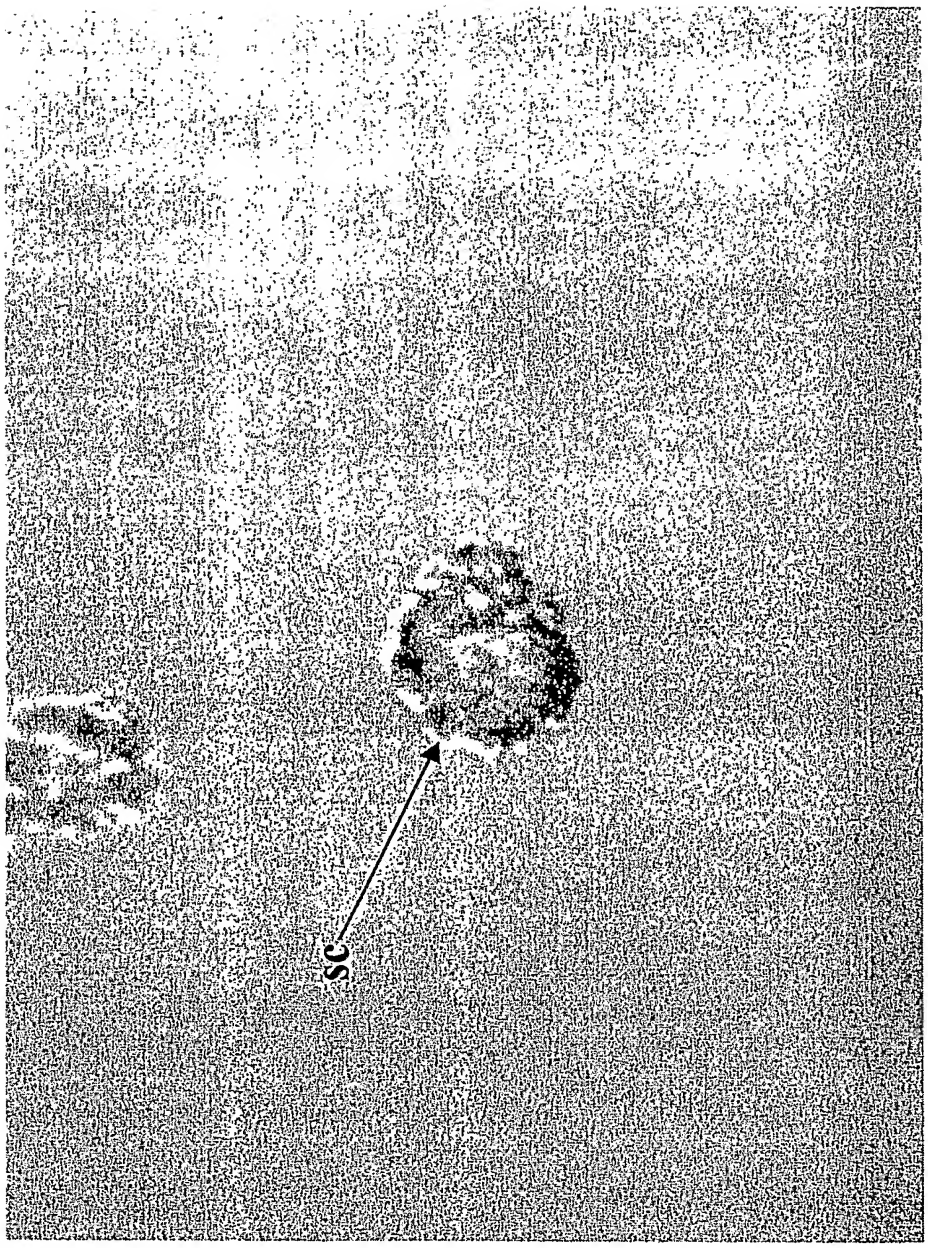


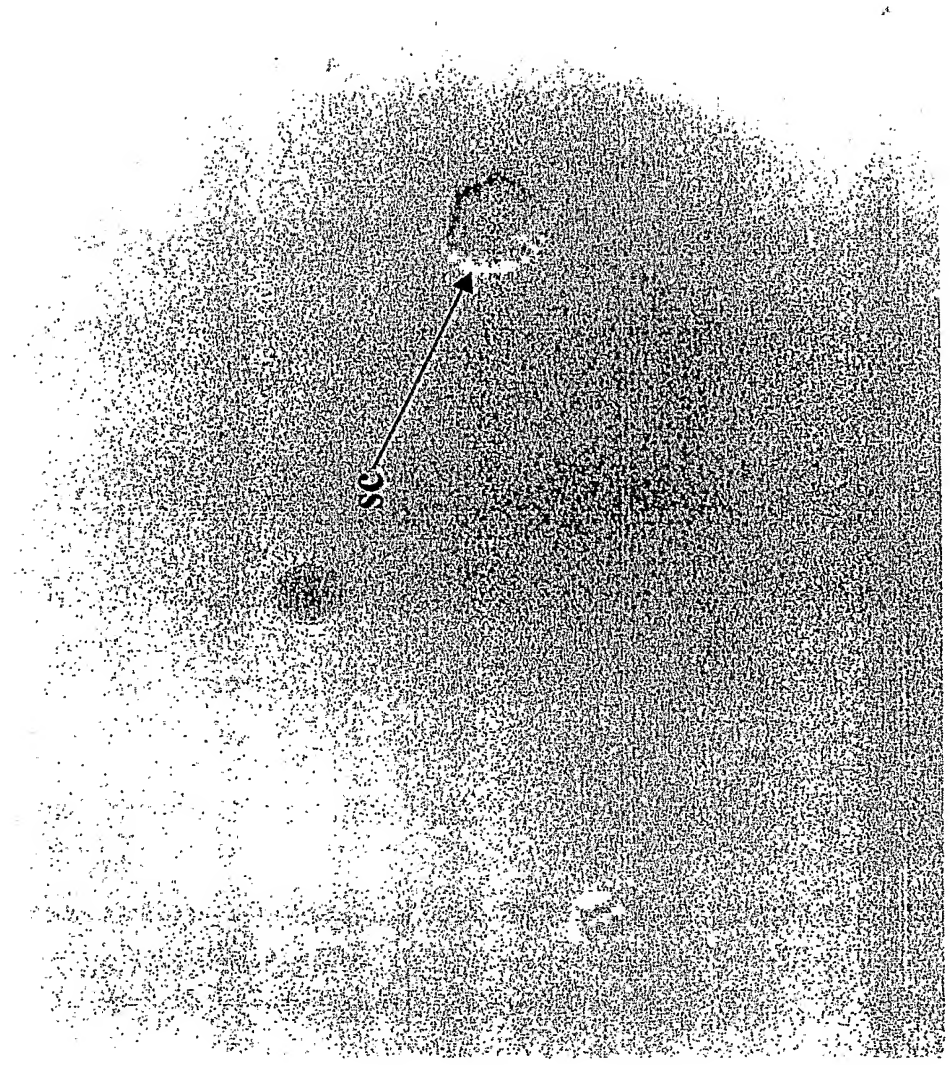
Fig. 5



a)

Fig. 6

Fig. 6



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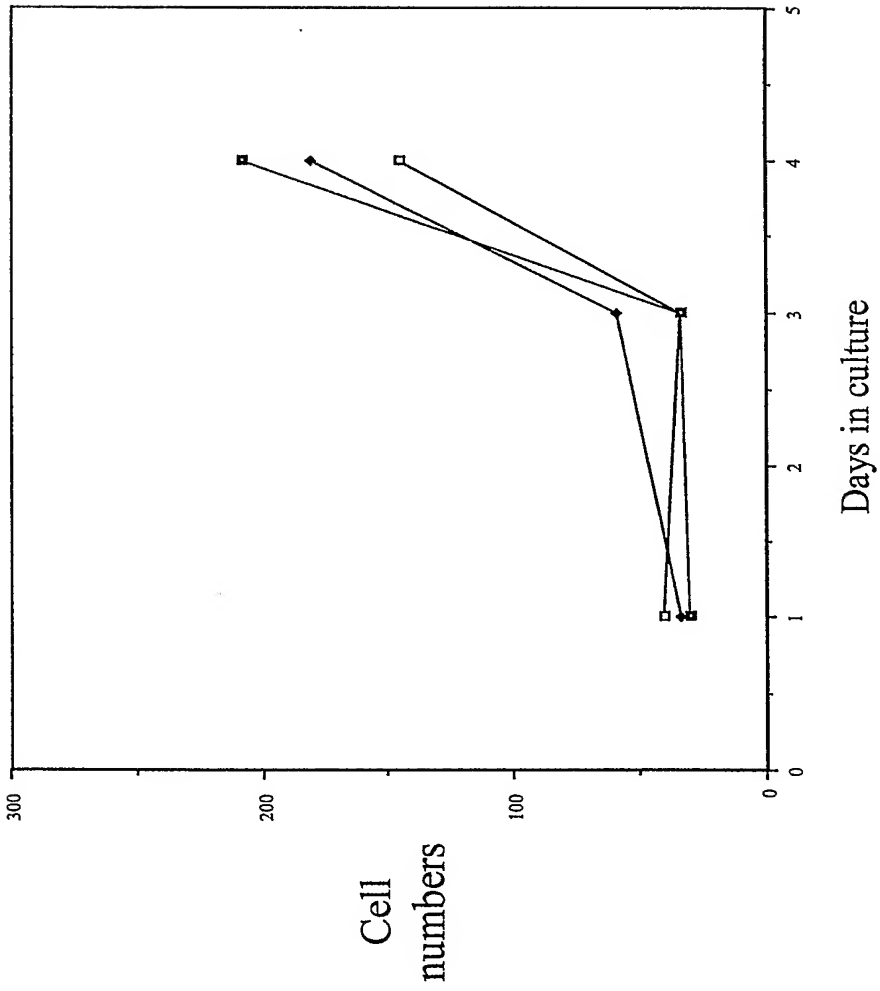

Fig. 7